CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY IN THE FIELD OF POLYPEPTIDE HORMONES[1]

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INTRODUCTION

Many of the polypeptides which exert a regulatory function within the animal organism may be termed classical hormones: they are elaborated within special glands, are increted into the blood stream, and are thus transported to their points of action. Other regulatory polypeptides are often designated as "tissue hormones"[2]. They originate in tissues whose physiological functions are not primarily hormone production (*e.g.* kidney, blood-plasma, brain, intestine, salivary gland *etc.*). Both types of compound will be included in this discussion.

It is with Sanger's[3] pioneering investigation into the structure of insulin (*Figure 1*) that organic chemistry has learned how to determine the number and sequence of amino-acid residues in polypeptide chains. This characteristic of a polypeptide is often called its *primary structure* and includes, of course, a detailed description of the configuration of each amino-acid residue. Depending on the nature of the molecular environment (neighbouring molecules in crystals, solvents, adsorbants, including cellular structures), the chains are known to be coiled not in a random, but in a very distinct, manner: conformations induced by hydrogen bonds within the polypeptide backbone (peptide bonds) are called *secondary structures*, those determined by forces between the amino-acid side chains (and amounting to a further folding of secondary structures) are called *tertiary structures*[4].

Although monumental examples for the determination of secondary and tertiary structures of protein molecules *in crystals* have been provided by X-ray examinations of the Cambridge group[5] (haemoglobin and cytochrome *c*), this method has not yet been applied to the few crystalline polypeptide hormones (many polypeptide hormones have not even been crystallized up to now, despite extensive purification, and, therefore, will not lend themselves to structure determination by this method).

Methods for the elucidation of three-dimensional architecture of polypeptides *in solution* are still in a rudimentary stage and may only yield information on certain details (*e.g.* hydrogen bonding of the phenolic —OH of tyrosine residues *etc.*)[6].

The contribution of the organic chemist to the study of structure–activity relationships in the field of polypeptide hormones is at present restricted to the correlation of primary structure (amino-acid sequence) to biological activity. This is not too great a drawback, however, as primary structure

certainly is by far the most important factor determining which three-dimensional shapes may possibly be assumed. Whether the shape of the hormone molecule in the act of entering a cell, of changing the permeability of membranes, or of reacting with other molecules (receptors) within a biochemical mechanism leading to a biological effect has anything in common with a preferred conformation in a crystal or in aqueous solution, is highly questionable. More probably, it is dependent on the spatial requirements of the particular cell wall constituents or receptor-molecules.

With these last statements we have entered the realm of the biochemist and biologist. Very much is known about over-all actions of hormones on organisms *in vivo* and tissues *in vitro*, but comparatively very little about the biochemical mechanisms which are influenced and the receptor-molecules involved. Some hormones seem to act upon the DNA of chromosomes (directly?), inducing particular genes to liberate their information, making it available for the synthesis of RNA and of specific proteins and enzymes[7]. Others may influence an enzyme system which is involved in the conversion of ATP into cycloadenylic acid, a process concomitantly stepping up the rate of glycolysis and TPNH-production which is essential for energetic and synthetic activities in the cell[8a]. In this latter case the specificity observed within a given set of hormones would be a function of different molecular characteristics not of the biochemical mechanism which is influenced, but of the cell walls which have to be transgressed.

Direct actions of hormones on enzymes are, however, still not unequivocally established (for a discussion see ref. 7). Further possibilities include interactions with cell membranes (or with membranes of sub-cellular structures) leading to specific changes of the permeability of such membranes which may account for the observed hormonal activity. A good example is provided by vasopressin which has been demonstrated to react with cell walls in a manner well defined by a number of chemical parameters[8b].

This leads to a series of basic questions: what are we really comparing when we are assaying two chemically distinct compounds in a given set of biological assays? Are we comparing their action on the same biochemical mechanisms, or are we triggering different ones which give the same observable biological effect? Are we certain that differences in resorption, enzymatic degradation and transport mechanisms (through cell walls) do not seriously affect our observations?

I would like to stress the point that the best we can do at present is to *compare the primary structure of polypeptides with over-all biological effects*. Our investigations are forestalled with great chemical and biological ignorance which may invalidate any conclusions on a molecular basis at which we might choose to arrive. The present approach has certainly been extremely successful in finding new products for therapeutical use, demonstrating its inherent ethical qualities. It is entirely insufficient, however, from the viewpoint of molecular biology, and a great amount of labour will be required before we shall be able to recognize the code contained in the amino-acid sequence and to understand the "languages" of the receptor-molecules.

At this point I should like to emphasize the important rôle which synthetic chemistry will have to play in this effort. Synthesis is the only means at hand to introduce chemical changes at will into particular parts of peptide

hormones and to fully determine the sequential requirements of a given hormonal activity.

AMINO-ACID SEQUENCES AND BIOLOGICAL FUNCTIONS OF NATURAL HORMONES

Insulin

The insulins of various species are composed of two peptide chains (A with 21 and B with 30 residues) linked together in a very specific manner by disulphide bonds to form two macrocyclic, heterodetic peptide rings. The intactness of this ring system is necessary for biological action[8, 9]. Insulin probably acts mainly upon cell walls, regulating the transport of glucose, amino-acids, fatty acids, sodium ions, *etc.*[10].

The changes in amino-acid sequence according to species (*Figure 1*) do not seem to alter the biological activity significantly, nor do they restrict it to the particular species. This may indicate, that certain amino-acids are "physiologically equivalent"[11] or that they occur in portions of the molecule that are not directly responsible for action (outside of an "active site"). Equal activity of these compounds may be the reason that these genetic changes have survived the process of evolution, whereas possible mutations affecting other parts of the molecule and impairing activity would have been lethal factors.

Different portions of the polypeptide chains of insulin actually do not seem to be equally responsible for activity: the "active site" must reside somewhere between the residues A_2, A_{21}, B_2 and B_{22} as determined by controlled enzymic degradation and chemical substitution of $-NH_2$ and $-OH$ groups[10]. The C-terminal aspartic acid of chain A is essential for activity, whereas 6 and 8 amino-acid residues of the B chain may be removed from the N- and C-terminals, respectively, without destroying activity[15].

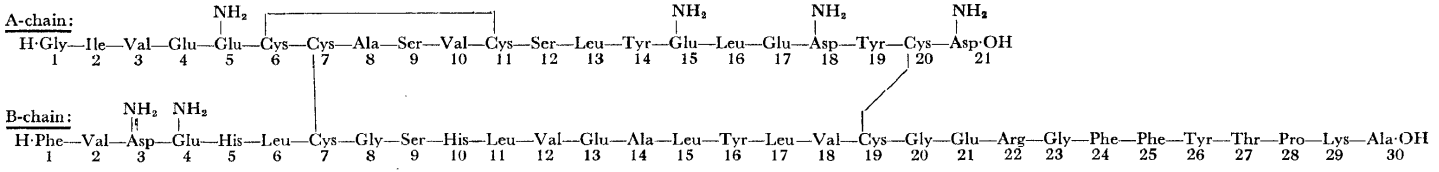
A number of speculations on secondary (α-helical[16, 17], β-extended[18]) and tertiary structures (dimerization[19]) have been reported. Until more evidence for their validity is produced, their bearing on the interaction between insulin and its hypothetical receptor-molecules cannot be assessed.

Another open question pertains to the rôle played by zinc for biological activity: zinc is known to be chelated rather strongly by insulin and it has been impossible to decide whether the active species is the chelate or the free insulin, because zinc is ubiquitous in biological fluids and could be picked up by insulin even during the course of an assay[20].

These findings probably represent the maximal information which can be gained with the type of methods employed, and further insight will have to await the *synthesis* of insulin and of analogues thereof with different chain length and different amino-acid sequence.

Glucagon

Glucagon seems to exert its main action on the metabolism of glucose. It liberates glucose from the liver and may be used to combat hypoglycaemic shock[21], it also enhances utilization of glucose in the cells[22]. Recent experiments point to a lipid-mobilizing activity of this hormone[22, 23]. It is at present impossible to make any relevant statements about structural requirements



Species	A-chain (sequence 8–10)	B-chain (C-terminal amino-acid)
	Ala—Ser—Val	—Ala·OH
	8 9 10	30
Cow	Thr—Ser—Ileu	—Ala·OH
Pig	Thr—Ser—Ileu	—Ala·OH
Sperm-whale	Thr—Ser—Ileu	—Thr·OH
Human	Thr—Gly—Ileu	—Ala·OH
Horse	Ala—Ser—Thr	—Ala·OH
Sei-whale	Ala—Ser—Val	—Ala·OH
Sheep		

Amino-acid residues are abbreviated essentially with the first three letters of their trivial name[12]. When written with a capital letter, they denote the natural stereoisomer, otherwise (lower-case letter) the unnatural (D-)isomer (glycine is abbreviated as Gly). Hetero-atoms of side-chain functions are not shown, substituents on such groups are located above or below the symbol for the amino-acid. They are meant to replace —H (in $—NH_2$, —OH, and —SH) or —OH (in side-chain —COOH). The same holds true for substituents on the α-NH_2 group (left-hand side of chain) and for α-COOH (right-hand side of chain), these substituents appearing at the sides of the amino-acid symbol. Thus, the symbol H·Cys·OH means L-cysteine, H·Cys·OH denotes hemi-L-cystine, H_2N(HOOC)CH—CH_2—S—CH(NH$_2$)(COOH)—CH_2—S—CH(NH$_2$)(COOH), and H·Cys·OH stands for L-cystine; H·Gly—Ala·OH is glycyl-L-alanine; Z·Gly—Ala·OCH$_3$ is carbobenzoxy-glycyl-L-alanine-methylester (Z— =  —CH_2OCO—, carbobenzoxy); H·Gly—Lys(Z)·OH would symbolize N^{α}-glycyl-N^{ϵ}-carbobenzoxy-L-lysine; Glu = L-glutamic acid; Glu(NH$_2$) = L-glutamine, *etc.*

Figure 1. Hormones of the pancreas: insulins of different species[12–14]

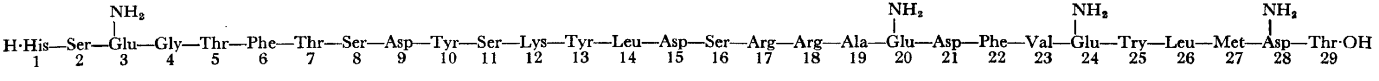


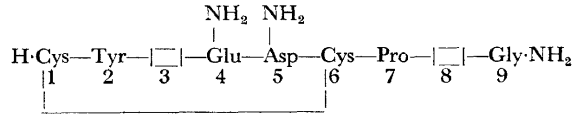
Figure 2. Hormones of the pancreas: glucagon (from porcine pancreas)[12, 13, 24]

for biological activity; all the fragments obtained by enzymic digestion seem to be inactive. Further knowledge will have to await alteration of the molecule at specific points by synthetic means.

Oxytocin, vasopressin, vasotocin

Whereas the hormones of the pancreas which influence carbohydrate metabolism in a somewhat antagonistic manner exhibit very great chemical differences, the posterior pituitary elaborates hormones which are chemically related to one another but produce dissimilar effects. We are faced with the fact that peptide hormones may be endowed with considerable specificity which is connected with structural details (for a discussion see refs. 25 and 26). Investigations in this field have been greatly stimulated and facilitated by synthetic approaches, the first ones being due to the school of du Vigneaud[27].

Typical for the chemistry of these hormones is a chain of 9 amino-acids with two residues of hemi-cystine in positions 1 and 6 joined by a disulphide bridge. The heterodetic macrocycle thus produced contains 20 atoms in the ring and is similar in size to the one encountered in the A-chain of insulin (residues 6 to 11).



Oxytocin from bovine [28,29], porcine[30], human [31], equine[32] and ovine[33] species: Ile(3) and Leu(8).
Vasopressin (*antidiuretic hormone*) from bovine (and many other) species[34]: Phe(3) and Arg(8); from porcine species[35]: Phe(3) and Lys(8).
Vasotocin (*osmolarity regulating hormone in cold-blooded vertebrates and birds*), first prepared by synthesis[36], then isolated from natural sources[37]: Ile(3) and Arg(8).

Figure 3. Hormones of the posterior pituitary: oxytocin, vasopressin, vasotocin[12, 13]

Oxytocin acts mainly on the smooth muscle of the uterus and the mammary gland, and is able to depress avian blood pressure. Pressor and antidiuretic activities are negligible. This pharmacological behaviour seems to be closely related to the presence of aliphatic amino-acid residues (isoleucine and leucine) in positions 3 and 8.

Exchange of these two residues for phenylalanine and arginine or lysine reduces the oxytocic properties very markedly, and effects on blood pressure and diuresis typical of *vasopressin* are produced.

We would expect the replacement of the non-polar amino-acid Leu(8) by a basic residue to be responsible for this strong change of activity. Nevertheless, the smaller difference between the non-polar amino-acids Ile(3) and Phe(3) also plays a definite rôle in determining the type of action. An analogue of oxytocin and vasopressin, *vasotocin*, containing Ile(3) of oxytocin and Arg(8) of vasopressin was first prepared by synthesis[36] and subsequently shown to be a compound regulating osmolarity in cold-blooded vertebrates and birds[37]. Its oxytocic activity is about 1/6 that of oxytocin and its pressor activity (rat) about 3 times less than that of Arg(8)-vasopressin[1, 13].

Many synthetic analogues of oxytocin and vasopressin have been prepared[27, 38]. From this work it can be concluded that activity depends on

the presence of a number of structural details, like size of the heterodetic ring, intactness of the C-terminal tripeptide chain *etc.*, again demonstrating a very appreciable degree of specificity which does not fall short of that displayed by other groups of compound, *e.g.* steroid hormones[25, 26].

Oxytocin was found to be a chelating agent for Cu^{2+}. An examination of the chelation properties (titration, spectra, comparison with copper-binding by desamino-oxytocin, Phe$^{(2)}$-oxytocin and simple glycine peptides) has given results which may be interpreted as indicating that the N-terminal amino group and 3 peptide nitrogens are the points of attachment of the metal ion. The chelate thus seems to be of the ordinary biuret-type[39].

Very little is known about the relationship between the conformation of oxytocin in aqueous solution and its biological activity. Ressler[40] has reported experimental conditions for producing a "denaturation" with concomitant loss of activity. Treatment with alkali results in intermolecular disulphide interchange which leads to inactive polymers, but the loss of activity after treatment with 7N urea at pH 8·0 and 4·3 (much less at 4·3) might be attributed to a change of conformation, because strong urea solutions are known to alter the three-dimensional structure of proteins. However, as the author states: ". . . the possibility of chemical change should be ruled out before this inactivation in urea is ascribed to change in the spatial configuration of the oxytocin molecule".

Adrenocorticotropin and the melanophore-stimulating hormones

Turning our attention to these hormones of the anterior (and intermediate) pituitary we again find (as in the case of oxytocin and vasopressin) striking similarities of chemical structure clearly differentiating them as a group from other hormones. The most obvious feature common to all of the hormones listed in *Figure 4* is the heptapeptide sequence -Met-Glu-His-Phe-Arg-Try-Gly- (included in the frame).

This heptapeptide sequence is partly responsible for one activity which all of these compounds (including ACTH) have in common and exert on the melanocytes of amphibia causing expansion of the melanophores and darkening of the skin[41–43]. A synthetic preparation of the heptapeptide in question (containing glutamine in place of glutamic acid)[44] exerts a high degree of corticotropin-releasing activity on isolated hypophyseal tissue *in vitro*[45] (this result has been confirmed with the heptapeptide containing the free γ-carboxyl of glutamic acid)[46]. It also displays an activity on melanophores[44] which is about 100,000 times less ($2{\cdot}8 \times 10^5$ U/g) in the *in vitro* assay[47] than purest preparations of α-MSH ($\sim 2 \times 10^{10}$ U/g)[48]. Similar low, but definite, activity is shown by a number of synthetic polypeptides containing the sequence His-Phe-Arg-Try-Gly (3×10^4 U/g)[49] or analogues thereof. The fact that (in this low range) activity is elicited also by analogues containing D-amino-acids (*e.g.* His-phe-Arg-Try-Gly, $3{\cdot}4 \times 10^5$ U/g)[50] would suggest that the "receptor" responsible for melanophore expansion reacts rather unspecifically against such high overdosage. Nevertheless, a great amount of work has been expended on the elucidation of the structural requirements of this activity (see ref. 1); many of the peptides examined are listed in *Figure 7*.

If the presence of α- and β-MSH in warm-blooded vertebrates is not only

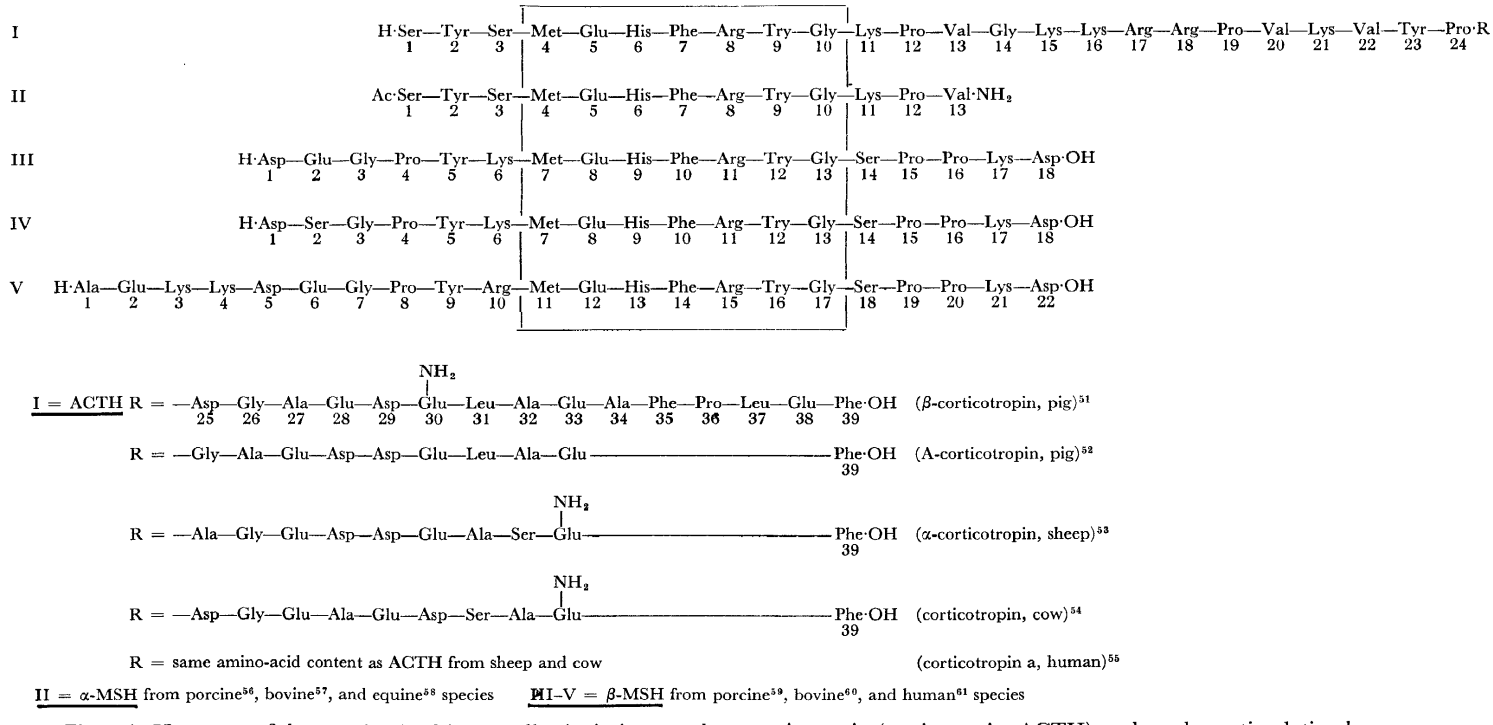
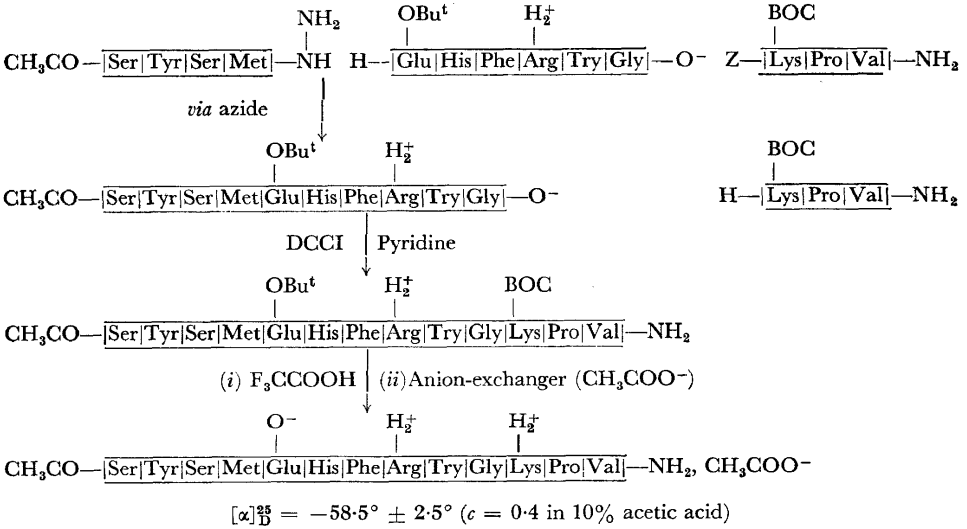


Figure 4. Hormones of the anterior (and intermediate) pituitary: adrenocorticotropin (corticotropin, ACTH), melanophore-stimulating hormones (α-MSH, β-MSH)[12, 13]

to be regarded as an evolutionary relict but as being physiologically meaningful, we will have to search for other activities of these compounds. Some possibilities are indicated by very recent investigations on β-MSH as a neural transmitter substance in cats[62] and on the action of α-MSH in stimulating the activity of the thyroid gland of guinea pigs[63].

For further studies it would be highly desirable to have an ample amount of synthetic material at hand, because it would be sure to be free of traces of other hormones which might be present in a preparation isolated from natural sources. A synthesis of α-MSH in small amounts has been reported[64]. The purity of this synthetic material, however, has been questioned[65]; the only preparation on a somewhat larger scale is that of an analogous, substituted tridecapeptide (*Figure 7*, No. II) which possesses full melanophoretic activity[65].

The demand for synthetic α-MSH can now be filled due to two new methods developed in my laboratories. They both utilize new combinations of protecting groups which may easily and selectively be split from the intermediates without causing damage to the peptide chain and loss of activity. The first one[67] (*Figure 5*) allows a straightforward synthesis of α-MSH utilizing intermediates and methods we had developed for the synthesis of β^{1-24}-corticotropin (see below). The second one[68] (*Figure 6*) yields an intermediate tridecapeptide with a free α-amino group which lends itself to the introduction of a variety of N^{α}-acyl groups besides acetyl. The yields are consistently good. In contrast to other preparations[64], activity of our synthetic material does not deteriorate on storage.



$[\alpha]_D^{25} = -58{\cdot}5° \pm 2{\cdot}5°$ ($c = 0{\cdot}4$ in 10% acetic acid)

Figure 5. Synthesis of α-MSH *via* BOC-protected intermediates[67, 69]*

* BOC = t-butoxycarbonyl; But = t-butyl; DCCI = dicyclohexyl carbodiimide.

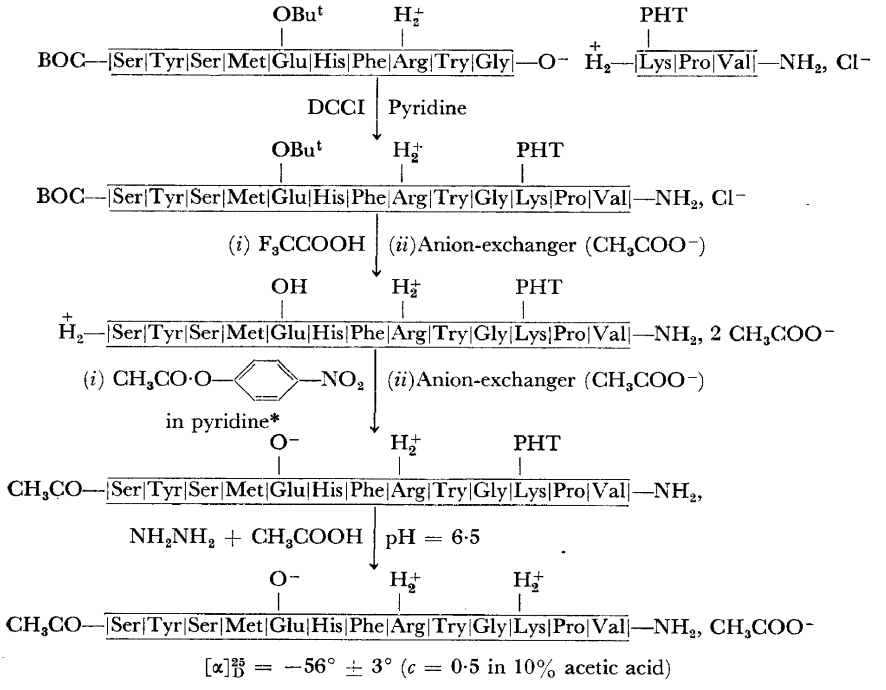


Figure 6. Synthesis of α-MSH *via* phthalyl-protected intermediates[68, 69]†

* Other acyl-groups (*e.g.* benzoyl) may easily be introduced in this step.
† PHT = phthalyl.

A protected derivative of bovine β-MSH (*Figure 7*, No. XII) has been synthesized[70]; although its protecting groups are not expected to be easily cleaved by biochemical means, it exhibits a rather high *in vitro* activity ($1{\cdot}4 \times 10^7$ U/g) as compared with β-MSH (2×10^9 U/g). A similar behaviour has been reported for the protected compound No. VIII (*Figure 7*). These observations are also indicative of a considerable unspecificity of the structural requirements for melanophore expansion. A complete synthesis of β-MSH has very recently been accomplished[70a].

ACTH—In this hormone structural elements of α-MSH (*viz.*, the tridecapeptide chain of α-MSH without the acetyl and amide groups) are combined with a C-terminal hexakosa-peptide chain (26 amino-acid residues in specific arrangements) to produce peptides with 39 residues. During their work on ACTH, Bell and his co-workers[51] were able to isolate, besides β-corticotropin (*Figure 3*), 7 other, chemically very similar, active compounds. It is not certain whether some of these are artefacts resulting from the isolation procedure, whether they simply reflect genetic differences of the population of swine from which the pituitaries were gathered, or whether they are meant to elicit qualitatively different hormonal activities.

The main biological action of corticotropin seems to be that on the adrenal cortex, both protein synthesis[78] and synthesis of specific steroids

No.		Ref.	(U/g)
	(*i*) *Peptides with a high activity* (down to ~1% of that of the corresponding natural product, α- or β-MSH) (*a*) *α-MSH and analogues; ACTH*		
I	Ac·Ser—Tyr—Ser—Met—Glu—His—Phe—Arg—Try—Gly—Lys—Pro—Val·NH_2 NH_2 CHO	48	$2 \cdot 10^{10}$
II	Ac·Ser—Tyr—Ser—Met—Glu—His—Phe—Arg—Try—Gly—Lys—Pro—Val·NH_2	65	$2 \cdot 10^{10}$
III	Ac·Ser—Met—Glu—His—Phe—Arg—Try—Gly—Lys—Pro—Val·NH_2 NH_2 Tos	71	$5 \cdot 10^{9}$
IV	Ac·Ser—Tyr—Ser—Met—Glu—His—Phe—Arg—Try—Gly—Lys—Pro—Val·NH_2	65	$2 \cdot 10^{9}$
V	Ac·Ser—Tyr—Ser—Met—Glu—His—Phe—Arg—Try—Gly—Lys—Pro—Val·NH_2 O ↑	66	$1 \cdot 10^{9}$
VI	H·Ser—Tyr—Ser—Met—Glu—His—Phe—Arg—Try—Gly—Lys—Pro—Val·NH_2	71	~$1 \cdot 10^{9}$
VII	H·Ser—Met—Glu—His—Phe—Arg—Try—Gly—Lys—Pro—Val·NH_2 NH_2 Tos	71	$1 \cdot 10^{8}$
VIII	Z·Ser—Tyr—Ser—Met—Glu—His—Phe—Arg—Try—Gly—Lys—Pro—Val·NH_2	65	$1 \cdot 10^{8}$
IX	H·Ser—Tyr—Ser—Met—Glu—His—Phe—Arg—Try—Gly—Lys—Pro—Val·R	73	$1 \cdot 10^{8}$
X	Ac·Ser—Tyr—Ser—Met—Glu—His—Phe—Arg—Try—Gly—Lys—Pro—Val·R	74	$8 \cdot 10^{8}$
	(*b*) *β-MSH and analogues*		
XI	H·Asp—Ser—Gly—Pro—Tyr—Lys—Met—Glu—His—Phe—Arg—Try—Gly—Ser—Pro—Pro—Lys—Asp·OH OCH_3 Tos NH_2 OCH_3	70a, 73	$2 \cdot 10^{9}$
XII	Z·Asp—Ser—Gly—Pro—Tyr—Lys—Met—Glu—His—Phe—Arg—Try—Gly—Ser—Pro—Pro—Lys—Asp·OCH_3	70	$1 \cdot 10^{7}$
	(*ii*) *Peptides with a low activity* (less than ~1% of the corresponding parent product)		
XIII	Ac·Ser—Tyr—Ser—Met—Glu—His—Phe—Arg—Try—Gly·OH	74a	$1 \cdot 10^{7}$
XIV	H·Ser—Tyr—Ser—Met—Glu—His—Phe—Arg—Try—Gly·OH	74a	$3 \cdot 10^{6}$
XV	H·Met—Glu—His—Phe—Arg—Try—Gly·OH NH_2	46	$1 \cdot 10^{6}$
XVI	Z·Ser—Met—Glu—His—Phe—Arg—Try—Gly·OH NH_2	75	$7 \cdot 10^{5}$
XVII	H·Ser—Met—Glu—His—Phe—Arg—Try—Gly·OH	75	$7 \cdot 10^{5}$

XVIII	H·His—Phe—Arg—Try—Gly—Lys(CHO)—Pro—Val·NH_2	75	$8 \cdot 10^6$
XIX	H·His—Phe—Arg—Try—Gly—Lys(Tos)—Pro—Val·NH_2	75	$5 \cdot 10^5$
XX	H·His—Phe—Arg(NO_2)—Try—Gly—Lys(CHO)—Pro—Val·NH_2	75	$5 \cdot 10^5$
XXI	H·Met—Glu(NH_2)—His—Phe—Arg—Try—Gly·OH	44	$3 \cdot 10^5$
XXII	H·Glu(NH_2)—His—Phe—Arg—Try—Gly·OH	44 49 72	$2 \cdot 10^5$
XXIII	H·Gly—His—Phe—Arg—Try—Gly·OH	76	$2 \cdot 10^5$
XXIV	H·His—phe—Arg—Try—Gly·OH	50	$3 \cdot 10^5$
XXV	H·His—Phe—Arg—Try—Gly·OH	49 75 77	$3 \cdot 10^4$
XXVI	H·His—Phe—Orn—Try—Gly·OH	77	$3 \cdot 10^4$
XXVII	H·His—phe—Orn—Try—Gly·OH	77	$3 \cdot 10^4$
	(iii) Peptides related to α-MSH with no activity		
XXVIII	Z·Met—Glu(NH_2)—His—Phe—Arg·OH	75	0
XXIX	H·Met—Glu(NH_2)—His—Phe—Arg·OH	75	0
XXX	Z·Ser—Tyr—Ser—Met—Glu(NH_2)—His—Phe—Arg·OH	75	0
XXXI	H·Ser—Tyr—Ser—Met—Glu(NH_2)—His—Phe—Arg·OH	75	0

Figure 7. Compounds with melanophore-stimulating activity (approximate comparison of activities on an *in vitro*[47] basis): Z = carbobenzoxy; R (Nos. IX, X) = residues 14–39 of ACTH (*Figure 3*); phe = D-phenylalanine (Nos. XXIV, XXVII)

(*Figure 8*) being enhanced[79]. The promotion of steroidogenesis (in particular hydroxylation) might partly (or wholly) be due to stepping up the conversion of ATP to cycloadenylic acid (adenosine-3′,5′-cyclophosphate, *Figure 9*)[8a].

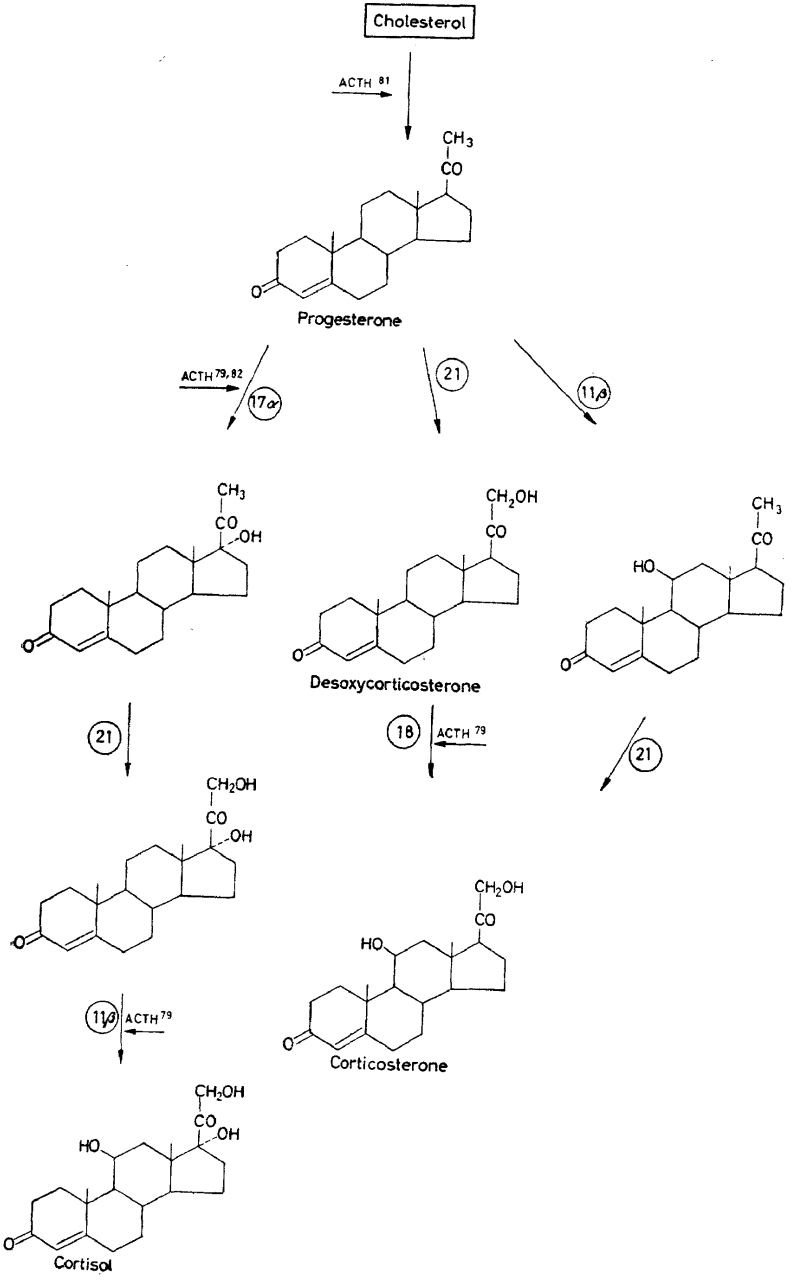


Figure 8. Proposed points of action of ACTH on the biosynthesis of C-21 adrenocortical steroid hormones[80]

All of these activities could be reasonably explained by an effect of ACTH on protein synthesis, including the synthesis of specific enzymes. This, together with the observation that ACTH causes an increase of the ribonucleic acid within adrenal cells[83], could be interpreted as an action of ACTH on the genes responsible for the synthesis of the proteins in question.

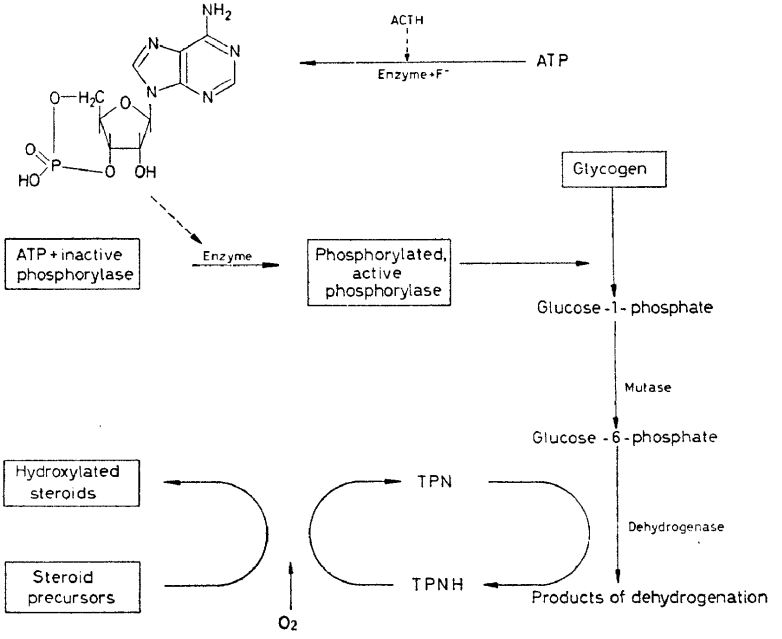


Figure 9. Proposed biochemical function of ACTH on hydroxylation of steroids[88]

Although a number of other effects (thymus-involution[84], lipid-mobilization[85] *etc.*) have been reported, the assay is usually based on the effects upon the adrenal cortex[86], and the results seem to be relevant to the clinical use of corticotropin. The biological potency of pure preparations of ACTH are estimated to be in the range of 80–150 I.U. per mg[87] (300 I.U. per mg has been reported[88] for Corticotropin-B).

Apparently, the N-terminal amino-acid (Ser) or amino-acids (Ser, Tyr) are necessary for biological activity[89], whereas C-terminal amino-acids are less so[90]. Biologically active peptides containing 28 and more amino-acid residues of the N-terminus have been isolated from peptic digests[51] and degradative evidence has been produced to suggest that a peptide containing the N-terminal 24 amino-acids displays a high degree of activity. It contains all of the basic residues of corticotropin, suggesting that these amino-acids are responsible for the activity measured.

Further insight into structure–activity relationships is strongly dependent on synthesis. *Figure 10* shows the peptides synthesized in the last two years by different groups of workers. Our own group has concentrated on developing new methods and combinations of protecting groups for peptide synthesis

Figure 10. Synthetic peptides with corticotropic activity

which allow the production of such peptides in especially good yields and in a most versatile manner. The synthetic products and further analogues will have to be tested in many different assay procedures in order to reveal qualitative and quantitative differences against ACTH. Before more work has been invested in this project, it is certainly too early to draw any definite conclusions.

Kallidin and bradykinin

The existence of bradykinin and its polypeptide nature have first been demonstrated by Rocha e Silva[97]. The pure nonapeptide may be isolated from plasma treated with trypsin[98] or with snake venom (Bothrops jararaca)[99]. The closely related decapeptide, kallidin 10 (one additional Lys at the N-terminal), is produced by the action of a specific enzyme, kallikrein, on plasma[100, 101]. The conversion of kallidin 10 to bradykinin (kallidin 9) is effected by a variety of enzymes occurring in plasma; *Figure 11* illustrates the interrelationship of the two peptides.

Pharmacological actions of the two peptides include action on smooth muscle (slow contraction of guinea pig ileum; oxytocic action at very low concentrations), lowering of blood pressure, enhancement of blood flow and capillary permeability, induction of activity of sweat glands, *etc.*[103, 104]. The relatively short duration of oxytocic action is due to destructive enzymes which may be inhibited by cysteine (destruction of angiotensin cannot be inhibited by this means)[105]. Physiological action may consist in local regulation of blood flow (*e.g.*, within the salivary gland).

Syntheses of kallidin 10[106, 107] and of bradykinin[108, 109] as well as of a number of analogues (*Figure 12*) have been reported. Octapeptide analogues of bradykinin having no Pro in position 7 (numbers according to kallidin 10) may be taxed as inactive (III is active only at a dose of 50,000 times that of bradykinin); however, when Pro$^{(4)}$ is missing, the octapeptide is only 100 times less active (isol. guinea pig ileum). C-terminal —Pro—Phe—Arg·OH seems to be essential for activity. Exchange of C-terminal —Arg·OH for citrulline and histidine (XI, XII), of N-terminal H·Arg— for citrulline (IX), and of both arginine residues for diaminobutyric acid or citrulline (VIII, X) lowers activity rather strongly, indicating a strong dependence of the activities tested on the presence of Arg in these positions. A complete reversal of sequence, leaving Arg$^{(2)}$, Pro$^{(4)}$, Phe$^{(6)}$, Pro$^{(8)}$ and Arg$^{(9)}$ in their original positions, eliminates activity (VII). The situation with Ser$^{(7)}$ is not clear as documented by a comparison of compounds (XIV, XV and XVI).

Activity of compounds XX and XXI (related to B 17–22 of insulin) has been taken as indicative of a particular *unspecificity* of peptides with biological activity[25b]. This argument loses much of its validity in view of the fact that peptides more closely related to bradykinin (*e.g.* III, VII, and others) elicit a similarly small amount of activity. Certainly, even if these compounds really do act upon the same receptors as the natural products, it is not permissible *a priori* to treat the response of a cell preparation to such overwhelmingly different concentrations (1:10^5 to 1:10^7) as being specifically related to the physiological one.

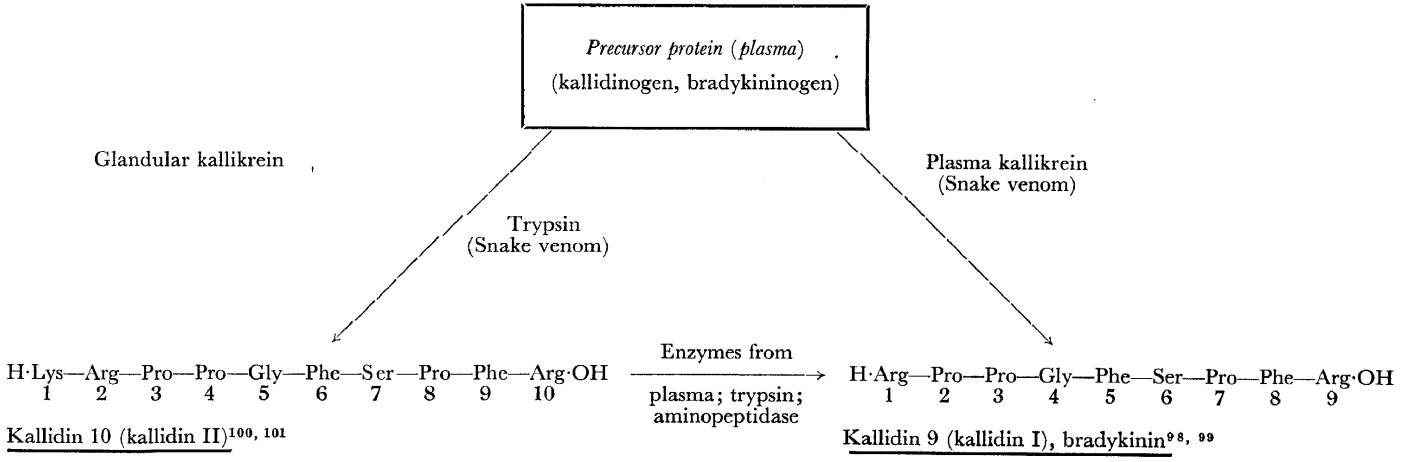


Figure 11. Tissue hormones of the kallidin–bradykinin type[12, 13, 102]

Angiotensin and hypertensin

The kidneys produce and store a specific enzyme called renin[119, 125], which is able to catalyse the cleavage of the decapeptide, angiotensin I, from a precursor protein (angiotensinogen) found in the α^2-globulin fraction of plasma[117–119]. This (apparently inactive) decapeptide is further degraded by a converting enzyme present in plasma[122] to the inactive dipeptide histidyl-leucine and the active octapeptide angiotensin II (hypertensin II). Species differences involve the exchange of Ileu$^{(5)}$ for the structurally closely related, and physiologically equivalent[11] Val$^{(5)}$ (*Figure 13*).

The isolation of angiotensin from blood is so difficult and gives such low yields that detailed studies of pharmacological and physiological action were only possible after the preparation of larger amounts by synthetic means[117]. A closely related synthetic derivative[117] with a higher rate of oxytocic to pressure action[126] has recently become available for therapeutical use (Hypertensin-CIBA®, No. II, *Figure 15*).

The biological action mainly studied until now is the enhancement of blood pressure[117, 118]. A number of clinical observations support the view that angiotensin is by far superior over catecholamines in the medication of shock of various origin (refs. 127–131 may serve to illustrate this point). Other effects of angiotensin include iron-incorporation (Fe$^{(59)}$) into red blood cells[132], and a qualitatively similar effect to that of vasopressin on water and salt excretion in *diabetes insipidus*[133, 134].

This latter effect leads us to one of a number of possible *physiological* actions of the renin–angiotensin system. It is believed that angiotensin serves to regulate kidney function as a locally acting tissue hormone[117–119, 125]. More recent evidence points also to a stimulation of the *zona glomerulosa* of the adrenals followed by enhanced excretion of aldosterone[125, 135]. This effect has been confirmed for human beings and is not produced by norepinephrin[136]. It is, however, too early to decide whether the stimulation of aldosterone production is a physiological effect of angiotensin or not.

Synthesis of analogues of angiotensin in the author's laboratories (*Figures 14* and *15*) and by Page and Bumpus and their co-workers (*Figure 16*) has given us a great amount of insight into the structural requirements for biological activity.

According to *Figure 14*, the asparaginyl$^{(1)}$ analogues of Val$^{(5)}$-angiotensin II (I) and of Val$^{(5)}$-angiotensin I (II) display an almost equal potency to enhance blood pressure in the nephrectomized rat. This is most probably due to cleavage of histidyl–leucine from II by the enzyme converting it to the active octapeptide (I). Obviously, if this explanation is acceptable, III can equally well be converted to I by this enzyme, whereas IV and V cannot (due to the inability of converting enzyme to split the Phe—Pro and the Phe—NH_2 bonds). Whether VI is cleaved by converting enzyme to an inactive form of I (with C-terminal D-phenylalanine) or not, is still a matter of question: in any case, D-phenylalanine in position 8 renders the decapeptide VI inactive.

Figure 15 lists some of the analogues of Val$^{(5)}$-angiotensin II prepared by the author's group. They were tested on the blood pressure of the nephrectomized rat by Gross and Turrian[140], by Paiva and Paiva on the blood pressure of the rat anesthetized with urethane and treated with hexamethonium

No.	*Residue No.* (kallidin 10)										(ng/ml)		(% of I)	
	1	2	3	4	5	6	7	8	9	10	*Isol. guinea pig ileum*	*Isol. rat uterus*	*Blood pressure*	*Ref.*
I		Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg	1	0·03	100	
II		Arg	Pro	—	Gly	Phe	Ser	Pro	Phe	Arg	100			110
III		Arg	Pro	Pro	Gly	Phe	Ser	—	Phe	Arg	50,000			111, 112
IV		Arg	Pro	Pro	Phe	Gly	Ser	—	Phe	Arg	Inactive at 2500			110
V		Arg	Pro	Gly	Pro	Phe	Ser	—	Phe	Arg	Inactive at 2500			110
VI		Arg	Pro	—	Gly	Phe	Ser	—	Phe	Arg	Inactive at 2500			110
VII		Arg	Phe	Pro	Ser	Phe	Gly	Pro	Pro	Arg	Inactive at 10,000	Inactive		113, 114
VIII		Dab	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Dab	Inactive			115
IX		Cit	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg		18*		114
X		Cit	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Cit		Inactive		114
XI		Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Cit			5	116
XII		Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	His			5	116
XIII		Arg	Pro	Pro	Gly	Phe	Ser Car |	Pro	F | Phe	Arg			100–150	116
XIV		Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg			1–10	116
XV		Arg	Pro	Pro	Gly	Phe	ser	Pro	Phe	Arg			40	116

XVI		Arg	Pro	Pro	Gly	Phe	Gly	Pro	Phe	Arg		0·03**		114
XVII	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg			66	116
XVIII	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	F-Phe	Arg			66	116
XIX					Leu	Val	Cys$_2$	Gly	Glu-NH_2	Arg	Inactive at 2,000,000			25b
XX					Leu	Val	Cys$_2$	Gly	Glu	Arg. NH_2	Active at 1,400,000	140,000		25b
XXI					Leu	Val	Cys$_2$	Gly	Glu-Arg		200,000	100,000		25b

Figure 12. Synthetic analogues of kallidin and bradykinin

Dab = Diaminobutyric acid (L)

Cit = Citrullin

F
|
Phe = *p*-Fluoro-phenylalanine

Car
|
Ser = *O*-Carbamyl serine

ser = D-Serine

* 1/600 of I^{114}

** As active as I^{114}

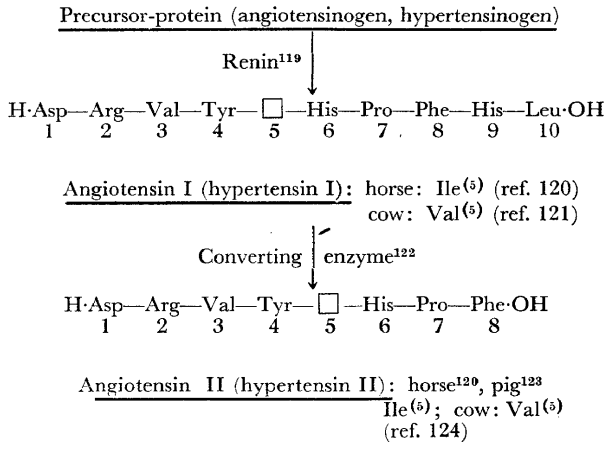


Figure 13. Tissue hormones of the angiotensin (hypertensin) type[12, 13, 117, 118]

bromide as well as on the isolated rat uterus (oxytocic activity)[141]. The asparaginyl analogue (II) is as active as Val(5)-angiotensin II (I) in the first pressure assay but elicits only about 50 per cent activity in the second one, indicating a methodical difference which has to be borne in mind when comparing the other data (from here onward, parallelism is satisfactory). The more pronounced difference in oxytocic activity between II and I is worth mentioning.

Alteration of position No. 1 reveals a rather low specificity connected with the first residue. Asp(NH_2) may be replaced by Gly or removed altogether without reducing activity by more than 50 per cent (III, IV). Most interesting compounds are V and Va, the D- and L- β-asparatyl analogues of I. I or II (*Figure 15*) may be converted to a mixture of diastereoisomers (V and Va) by transpeptidation and partial racemisation of Asp(1). This is achieved by heating aqueous solutions of I or II at 100° (*Figure 17*). Unexpectedly, the pressure activities of both β-compounds are considerably higher than that of the natural product (I). This is probably due to retarded degradation by plasma enzymes [142b].

Position No. 2 reveals greater specificity. The hexapeptide (VI) is almost inactive, a finding at variance with that on a similar peptide in the Ile(5)-angiotensin II series (*Figure 16*, No. V). It is surprising to see that it is probably not only the basicity connected with the side chain of arginine which is necessary for producing activity, but that a steric element also is involved (nitrogen on the δ-carbon atom ?). This statement is suggested by the strong activities of VII (not appreciably basic, due to nitro-substitution) and VIII (nitrogen on δ-C) as compared to IX (nitrogen on ϵ-C). The comparison seems to be permissible in spite of the fact that IX contains Leu in position 5, because XI is reasonably active.

Turning to the *two residues with hydrophobic side chains*, Val(3) and Val(5), we find indications of a certain difference of specificity: the Leu(3) analog is

Comp. No.		*Amino-acid residue no.*										*Activity (blood pressure of the nephrectomized rat)*[138]
		1	2	3	4	5	6	7	8	9	10	
I	H·	NH_2 | Asp | NH_2	Arg	Val	Tyr	Val	His	Pro	Phe	·OH		100
II	H·	NH_2 | Asp | NH_2	Arg	Val	Tyr	Val	His	Pro	Phe	His	Leu ·OH	50–100
III	H·	NH_2 | Asp | NH_2	Arg	Val	Tyr	Val	His	Pro	Phe	His	Leu ·NH_2	50–100
IV	H·	NH_2 | Asp | NH_2	Arg	Val	Tyr	Val	His	Pro	Phe	Pro	Phe ·OH	0·2
V	H·	NH_2 | Asp | OH	Arg	Val	Tyr	Val	His	Pro	Phe	NH_2		3
VI	H·	Asp	Arg	Val	Tyr	Val	His	Pro	phe	His	Leu ·OH (ref. 139)	0

↑ (Point of action of converting enzyme)

Figure 14. Synthetic analogues of Val(5)-angiotensin I[117, 137]

fully active (XII), whereas a significant decrease was observed with the Leu(5) analogue (XI).

Concerning position 4, our conclusions are different from those of Page and co-workers[143]. These authors state that one of the minimal requirements for activity is a tyrosine residue in position 4. This was deduced from a comparison of the two compounds Nos. V and VII (*Figure 16*). The hexapeptide (V, *Figure 16*) showed a higher activity than our hexapeptide (VI, *Figure 15*); this activity was, despite its being only 2·3 per cent of the activity of I (*Figure 16*), assumed to be significant and characteristic of the receptor; replacement of Tyr(4) by Phe(4) (as in VII, *Figure 16*) produced complete inactivation, suggesting that the hydroxyl group was of greatest importance. It seems to be dangerous, however, to draw conclusions from quantitative differences at such a low level of activity, as surmised by the fact that the corresponding Phe(4) analogue of angiotensin II (XIII, *Figure 15*) elicits full 10 per cent of the activity of the Tyr(4) compound (II, *Figure 15*). This clearly demonstrates that the –OH group of Tyr(4) is *not* a minimal requirement.

Turning again to *Figure 15*, we find that chain length is also essential, an additional tyrosine residue next to the original one lowering activity very appreciably (XIV). How essential steric details are, is demonstrated by XV which is inactive because of the replacement of L-tyrosine (Tyr) by its D-isomer (tyr)[144].

Alteration of position no.	Compound no.	Amino-acid residue no.										Activity relative to compound No. I		
												Pressure (rat)		
			1	2	3	4	5	6	7	8		Nephrectomized	Urethane anaesthet. + hexamethonium[141]	Isol. rat uterus[141]
①	I	H·	Asp	Arg	Val	Tyr	Val	His	Pro	Phe	·OH	100	100	100
	II	H·	Asp	Arg	Val	Tyr	Val	His	Pro	Phe	·OH	100	53±4	27±3
	III	H·	Gly	Arg	Val	Tyr	Val	His	Pro	Phe	·OH	50	36±6	22±2
	IV		H·	Arg	Val	Tyr	Val	His	Pro	Phe	·OH	50	26±4	23±3
	V	H·	Asp	–Arg	Val	Tyr	Val	His	Pro	Phe	·OH	150 (ref. 142)		
	Va	H·	asp	–Arg	Val	Tyr	Val	His	Pro	Phe	·OH	150		
②	VI			H·	Val	Tyr	Val	His	Pro	Phe	·OH	<0·1	<1	<0·1
	VII	H·	Asp	Arg	Val	Tyr	Val	His	Pro	Phe	·OH	50	25±3	3·1±0·2
	VIII	H·	Asp	Orn	Val	Tyr	Val	His	Pro	Phe	·OH	20	8·9±0·9	1·6±0·2
	IX	H·	Asp	Lys	Val	Tyr	Leu	His	Pro	Phe	·OH	<1	—	—
③+⑤	X	H·	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	·OH	100	52±8	33±3
	XI	H·	Asp	Arg	Val	Tyr	Leu	His	Pro	Phe	·OH	25	—	—
	XII	H·	Asp	Arg	Leu	Tyr	Val	His	Pro	Phe	·OH	100	—	—
④	XIII	H·	Asp	Arg	Val	Phe	Val	His	Pro	Phe	·OH	10	4·9±0·8	2·5±0·4
	XIV	H·	Asp	Arg	Val	Tyr_2	Val	His	Pro	Phe	·OH	1	0·33±0·5	0·10±0·02
	XV	H·	Asp	Arg	Val	tyr	Val	His	Pro	Phe	·OH	0	—	—
⑥	XVI	H·	Asp	Arg	Val	Tyr	Val	x* His	Pro	Phe	·OH ref. 145)	—	<1 (ref. 145)	<1 (ref. 145)
⑧	XVII	H·	Asp	Arg	Val	Tyr	Val	His	Pro	Phe Br	·OCH_3	10	—	—
	XVIII	H·	Asp	Arg	Val	Tyr	Val	His	Pro	Phe	·OH	50	—	—
	XIX		H·	Arg	Val	Tyr	Val	His	Pro	phe	·OH	0·2	—	—
	XX	H·	Asp	Arg	Val	Tyr	Val	His	Pro	·OH		0·05	—	—

x
* His = Product of photo-oxidation of the histidyl-residue.

Figure 15. Synthetic analogues of Val(5)-angiotensin II[117, 137]

The São Paulo group[145] has subjected our II to photo-oxydative inactivation, destroying the imidazole portion of the *His*$^{(6)}$*-residue*: activity is also abolished (XVI, *Figure 15*).

Whereas nothing is known about the *specificity of position 7* (Pro), we do know that phenylalanine with a free carboxyl group is an essential feature of *position 8*. Amide formation (V, *Figure 14*) and esterification (XVII, *Figure 15*) reduces activity to 3 per cent and 10 per cent, respectively. Compound XX (*Figure 15*) which lacks Phe$^{(8)}$ and compound IX (*Figure 16*) in which Phe$^{(8)}$ is replaced by Ala$^{(8)}$ are both inactive; this is also true for XIX (*Figure 15*) which has D-phenylalanine in place of the L-isomer and shows only 0·2 per cent activity compared with 50 per cent for the heptapeptide IV (*Figure 15*). Even such a slight alteration as substitution by bromine in the *p*-position of the phenyl ring reduces activity to 50 per cent (XVIII, *Figure 15*).

Alteration of position no.	*Compound no.*	*Amino-acid residue no.*										*Activity relative to I*	
			1	2	3	4	5	6	7	8		*Pressor (anaesthet. rat, hexamethonium)*	*Isol. rat uterus*
①	I	H·	OH | Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	·OH	100	100
	II*		Suc	Arg	Val	Tyr	Ile	His	Pro	Phe	·OH	60	51
	III		H·	Arg	Val	Tyr	Ile	His	Pro	Phe	·OH	21·6	18
	IV	H·	Arg	Arg	Val	Tyr	Ile	His	Pro	Phe	·OH	14·7	69
①–③	V		—	H·	Val	Tyr	Ile	His	Pro	Phe	·OH	2·3	6
	VI		—	—	H·	Tyr	Ile	His	Pro	Phe	·OH	0	0
①, ② + ④	VII		—	H·	Val	Phe	Ile	His	Pro	Phe	·OH	0	0
	VIII		—	H·	Val	Ala	Ile	His	Pro	Phe	·OH	0	0
⑧	IX	H·	OH | Asp	Arg	Val	Tyr	Ile	His	Pro	Ala	·OH	0	0

* Suc = succinyl.

Figure 16. Synthetic analogues of Ile$^{(5)}$-angiotensin II[118,143]

The compounds of *Figure 16* have been prepared by Page *et al.*[143]. Most of the results have just been discussed. The considerations applying to the comparison of VII with V are also valid for VIII. Interesting is the strong enhancement of oxytocic activity over pressor action in the Arg$^{(1)}$-analogue (IV). It seems possible that this compound would activate similar receptors as are activated by kallidin 10 and kallidin 9.

Conformational aspects of angiotensin—Experiments on reversible denaturation (change of optical rotation) and concomitant loss of activity by treatment with strong solutions of urea and arginine have been reported[143, 146]. Whether these observations reflect a change of conformation (from a biologically active to an inactive conformational isomer) or are simply due to strong adducts with urea or arginine which decompose only very slowly on dilution (the released quantities being quickly degraded by enzymes before becoming active) is still a moot point. The hydroxyl group of tyrosine has been shown

to dissociate with a normal pK-value and normal heat and entropy of dissociation[146] which seems to exclude the participation in any intramolecular hydrogen-bonds stabilized by special conformations.

Metal binding[147]—The α- and β-Val$^{(5)}$-angiotensins, Asp(NH$_2$)$^{(1)}$, Val$^{(5)}$-angiotensin, and Gly$^{(1)}$, Val$^{(5)}$-angiotensin give quantitative, amorphous

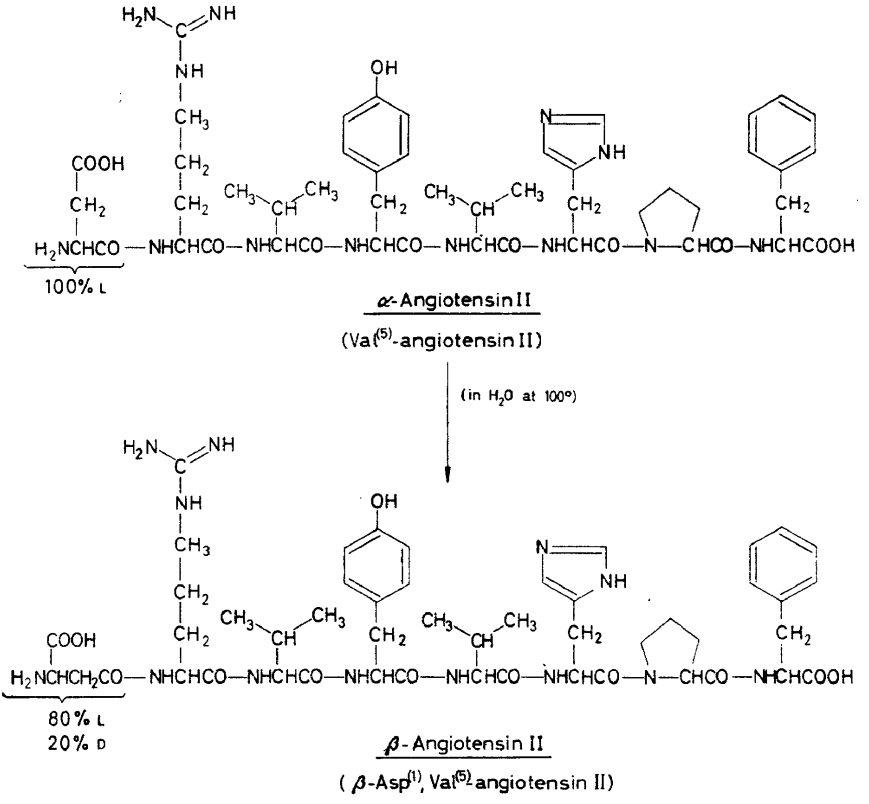


Figure 17. Conversion of α- to β-angiotensin II[142]

precipitates with Cu^{2+} and Zn^{2+} salts in aqueous solution. Analysis shows that 1 atom of the metal is bound to 1 molecule of angiotensin. Despite the precipitate, the mixture can be rather reproducibly titrated (*Figure 18*), showing that chelation is accompanied by a stepwise loss of protons: at first 2 are liberated (Zn^{2+}, Cu^{2+}), these are followed by 2 (Cu^{2+}) or 3 (Zn^{2+}) other protons. The Zn-chelate is active like angiotensin when applied intraveneously. As in the case of insulin, one might debate whether angiotensin acts upon the receptor in the "free" or in a chelated state, because metal ions could be picked up in the body.

Chelation has also been observed with other metal ions, *e.g.* Fe^{3+}, Co^{2+} *etc.*[147] and might possibly have some connection with the iron-transport activity[132] of Asp(NH$_2$)$^{(1)}$, Val$^{(5)}$-angiotensin.

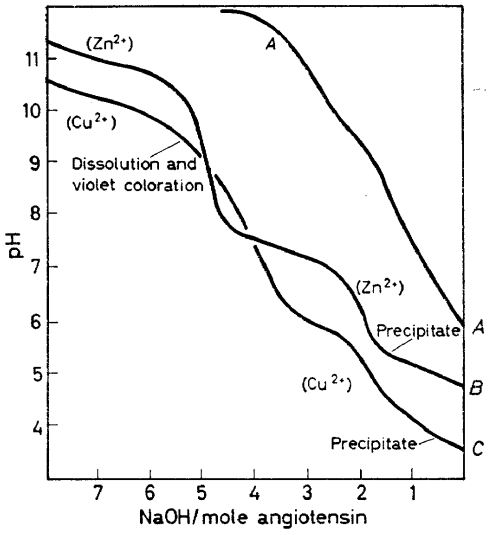


Figure 18. Titration of β-Val(5) angiotensin II with NaOH (*A*); after addition of 2 moles of ZnCls (*B*); after addition of 2 moles of $CuSO_4$ (*C*) (Titrigraph, Radiometer A.B., Kopenhagen)[147]

Substance P

Extracts of intestine and brain showing neurotropic and musculotropic activities have been prepared by von Euler and Gaddum[148a]. These extracts have been partially purified[148b]; the active compounds appear to be peptides. Recent work has led to a further purification of the musculotropic peptide from intestine[103, 149–151]; the most active preparation has been isolated from bovine brain and contains (in agreement with the results of Vogler[150]) 13 different amino-acids[151]. This is at variance with the findings of Franz *et al.*[149]. The musculotropic action of the peptide isolated from brain is easily distinguishable from that of bradykinin: it contracts the isolated guinea pig ileum and hen's coecum (threshold dose 0·5 ng/ml), the isolated rat uterus only in doses 100 to 200 times larger, and lowers the blood pressure of the rabbit (bradykinin is inactive on the hen's coecum[101], its relative activities on guinea pig's ileum and rat's uterus are inverse). The pair bradykinin–substance P is another example of the high specificity of polypeptides.

Intestine, brain

↓ Extraction[148]

Polypeptide

(Lys, Arg, Asp, Ser, Glu, Pro, Gly, Ala, Leu/Ile, Phe)	30,000 U/mg, from intestine[149]
(Lys, Arg, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile/Leu, Phe)	100,000 U/mg, from intestine[150]
(Lys, Arg, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Phe)	150,000–300,000 U/mg, from brain[151]

Figure 19. Musculotropic factor of substance P [103, 152]

CONCLUDING REMARKS

Biological activity of polypeptides is apparently very specifically related to structure and configuration of these compounds (primary structure). It remains to be demonstrated whether particular conformations (secondary and tertiary structures) which are stable in the solid state or in solution are necessary for activity. However, to be able to assume a conformation demanded by the receptor, certain features of primary structure must needs be present.

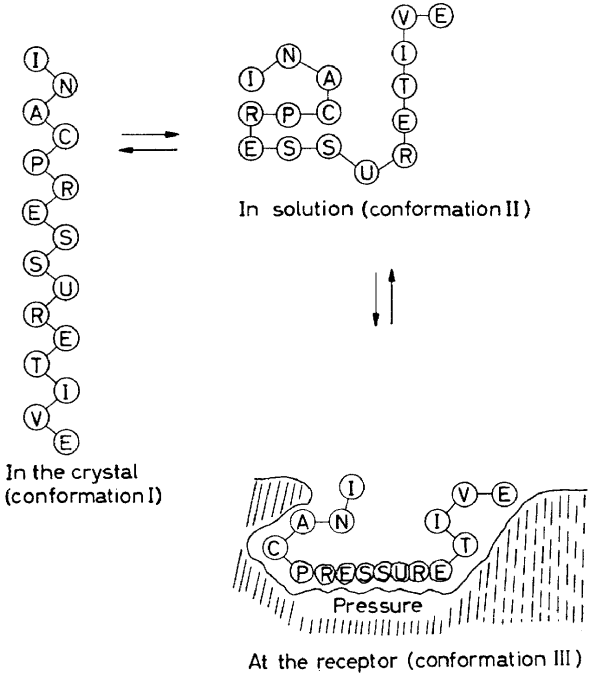


Figure 20. Possible conformational changes, active and inactive sites in polypeptide hormones. The conformation I in the crystalline phase might be completely different from conformation II in solution. Here, the " inactive " portion (sympolized by the letters INACTIVE) might serve a number of purposes, *e.g.* solubilization, *etc.* Conformation III at the receptor site may be still different and mainly determined by the active site (PRESSURE) interacting with a particular chemical structure (pressure) of the receptor—the inactive portions playing minor rôles.

The chains of biologically active polypeptides contain portions which are essential for activity, others which are not. The correct amino-acid sequence within the *active site* seems to be necessary to activate the right receptors, although variations at certain points appear to be more permissible than at others (specificity requirements of particular positions in angiotensin II probably become more exacting in the following sequence: $Asp^{(1)} \leqslant Val^{(3)} < Val^{(5)} < Arg^{(2)} \leqslant Tyr^{(4)} \leqslant Phe^{(8)}$). Chain length outside of the active site sometimes is necessary for producing quantitatively a maximum effect: these "inactive" sites probably also contribute to transport properties, to reduction of enzymatic viability *etc.* The "inactive" site probably has to

depend on specific amino-acid sequences to be able to fulfil its task, although the requirements might be less stringent.

The examination of sequence–activity relationships must be carried out at a high level of activity; this is the only way to be reasonably sure that one is dealing with specific effects and not with non-specific ones (a point that has often been neglected).

It is very tempting to compare amino-acid sequence with other linear codes, for example with a sequence of letters in written language. Whether a "language" understandable for all receptors will issue from our endeavour, or whether every receptor or certain groups of receptors have different "languages", is rather irrelevant at the moment, but adds quite an amount of intellectual incentive to the problems presented here.

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